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13. ABSTRACT (Maximum 200 words) The goal of this project is to develop Bowman-Birk protease inhibitor (BBI), a soybean polypeptide, as a chemopreventive agent for breast cancer. In order to achieve this goal, we proposed the following two specific aims in our original application: (1) using an <i>in vitro</i> mammary gland culture system to demonstrate the anti-transformation activity of both BBI and its palmitic acid conjugate (Pal-BBI), and (2) using a mouse model to demonstrate the advantages of Pal-BBI in oral delivery of BBI. During the past year, we have made three critical findings that are consistent with our hypotheses. First, we found for the first time that BBI can prevent the transformation of mammary glands induced by the treatment of a chemical carcinogen, 7,12-dimethylben[a]anthracene (DMBA). A 66% decrease of transformation incidence was observed in the presence of 20 µg/ml of BBI in the transformation assay medium. Secondly, we found that Pal-BBI was at least as effective as BBI in the chemopreventive assay. Thirdly, we found that Pal-BBI is significantly more stable than BBI in the gastrointestinal (GI) tract of the mouse, suggesting that the lipidized polypeptide is a better candidate for the design of an oral formulation for the chemoprevention of breast cancer. These results strongly support our original proposal, and will be very useful for the development of an effective and practical chemopreventive agent for breast cancer.				
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FOREWORD

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(5) INTRODUCTION

This second annual report covers the progress of the project, "Breast Cancer Prevention by a Soybean Protein", from July 15, 1997 to July 14, 1998. In the original application, we proposed to investigate chemopreventive effects of Bowman-Birk protease inhibitor (BBI) and its palmitic acid conjugate (Pal-BBI) on breast cancer. The technical objectives in the original proposal were: (a) to demonstrate breast cancer preventive effect of BBI or Pal-BBI by using mouse mammary gland cultures, and (2) to obtain preliminary results on the oral delivery of Pal-BBI in mice. During our first year, progress was made in the preparation of Pal-BBI, establishment of mouse mammary gland cultures for the transformation assay, and initial study of oral delivery of BBI and Pal-BBI.

During the second year, we have encountered a contamination problem in mammary gland cultures which, as we eventually identified, was caused by the filter unit of the O₂-CO₂ gas line for the gland culture chamber. This contamination problem has significantly delayed our progress, and, consequently, we have requested and obtained a no-cost extension of this two-year project for one more year, until July 14, 1999.

In this report, results from the continuation of pursuing the two original technical objectives during the past 12 months will be discussed. In addition, studies addressing the critiques from the reviewers of the first year progress report will also be presented.

(6) BODY

I. Technical Objective 1

1. Comparing the transformation of cervical and thoracic mammary glands in culture

Because we used both the first (cervical) and the second (thoracic) pairs of mammary glands in our tests, the reviewers were concerned whether there was a difference between these two sets of glands in the transformation assay. To clarify this issue, we compared results obtained from cervical and thoracic mammary glands from same group of mice in their responses to DMBA-induced transformation. As shown in Table 1, the differences between these two sets of glands in DMBA-induced transformation and BBI-mediated chemoprevention are statistically insignificant. However, we agree with the reviewers' suggestion that, in all of the transformation experiments, the mammary glands from each mouse were split equally into the treated and the control groups so that each group consists of same number of the cervical and thoracic glands.

2. Comparison the chemopreventive effects of BBI and Pal-BBI in DMBA-induced transformation of cultured mouse mammary glands (Task 4, 5, and 6)

The transformation of cultured mouse mammary glands induced by the chemical carcinogen, DMBA, was carried out as described in our original proposal. In a typical transformation assay, cultured mammary glands were divided into two groups, i.e., the control and the transformation groups. Mammary glands in the control group were treated with only DMSO, the solvent of DMBA; and those in the transformation group were treated with various concentrations of DMBA in DMSO. Mammary glands were treated on day 3 and 4 only and then cultured in promoting medium and lactogenic medium for a total of 10 days before changed to regression medium. After 14 days in the regression medium, mammary glands were fixed, washed, and then stained in Alum Carmine overnight. After dehydration, the stained glands were mounted on slides and examined under a dissecting microscope. The nodule-like alveolar lesions (NLAL) were identified as

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described in our previous annual report (1996-1997). When BBI and Pal-BBI were tested for their chemopreventive effects, they were included in both the promoting and lactogenic medium, but not in the regression medium.

Results obtained from the transformation assays are summarized in Table 2. Several factors were investigated in order to optimize the transformation assay. The dose of 2 µg/ml was chosen for DMBA because it was the highest concentration without significant toxicity. In addition, 2 µg/ml of DMBA also showed a higher transformation incidence than that of either 3 or 4 µg/ml of DMBA in the assay, possibly due to a low toxicity. Table 2 also shown that BBI is capable of preventing DMBA-induced transformation in cultured mammary glands. A BBI concentration of 20 µg/ml was chosen because it is the lowest concentration of BBI showing a significant prevention on DMBA-induced transformation. This concentration is comparable to those used in studies of chemical carcinogen-induced transformation in C3H10T1/2 cells (1). Currently, we are working on the Task 6, i.e., to test Pal-BBI in mammary gland transformation assay. We have obtained results from the treatment of 20 µg/ml of Pal-BBI by using same exposure time as in the treatment of 20 µg/ml BBI in the transformation assay. As shown in Table 2, Pal-BBI and BBI have an identical chemopreventive effect at this concentration. This result indicates that Pal-BBI is at least as effective as BBI in the prevention of the DMBA-induced transformation of mammary glands. The treatment of mammary glands at lower Pal-BBI concentrations, as well as with a shortened exposure time, will be compared with that of BBI in the coming year.

II. Technical Objective 2

1. Biodistribution of orally administered BBI and Pal-BBI in Balb/c mice

The biodistribution and pharmacokinetic studies were carried out in BALB/c mice, rather than in CF-1 mice as proposed in our original proposal. This change was suggested by the reviewers of the first annual report in order to be consistent with the same strain of mouse used in the transformation assays. In order to verify previous results, a study was carried out to compare the pharmacokinetics and biodistribution of intravenously injected BBI and Pal-BBI in Balb/c with data obtained previously from CF-1 mice. It was found that *iv*-injected Pal-BBI in BALB/c mice can achieve (a) a prolonged plasma half-life of Pal-BBI, (b) an increase of liver absorption of BBI, and (c) a decrease of kidney elimination of BBI. These results are identical to those obtained in CF-1 mice as described in our previous report (2).

2. Oral delivery of BBI and Pal-BBI in Balb/c mice (Task 5 and 6)

Female BALB/c mice, 7-8 weeks old, were fasted for 16-hr and, subsequently, were fed with either ¹²⁵I-BBI or ¹²⁵I-Pal-BBI using a gavage needle. The doses and radioactivities of BBI and Pal-BBI were identical, i.e., 3 mg/kg in 1% Intralipid-PBS and 5x10⁶ cpm per mouse. Animals were sacrificed at 0.5, 1.5 and 3 hr after the feeding, and the following organs were removed and counted in a gamma counter: blood, liver, kidneys, small intestine, large intestine, and stomach. There was no statistically significant difference between BBI and Pal-BBI in blood, kidneys and liver, because the amounts localized in these organs were too low to be determined. However, there were significant differences in the localization in the GI tract. As shown in Table 3, the retention of Pal-BBI in stomach was strikingly longer than that of BBI. The prolonged stomach retention

Table 1
Transformation effects in first pairs and second pairs of mammary glands

	Total Nodules/total glands*		Glands with nodules/total glands**	
	First pairs	Second pairs	first pairs	second pairs
Control	1	0	1/10	0/10
DMBA 2µg/ml	12	11	5/5	5/5
BBI 20 µg/ml	1	2	1/12	1/12
DMBA+BBI	7	7	6/15	4/13

First pairs of glands compare with second pairs of glands by chi-square test:

*p= 0.716634

**p= 0.9918354

Table 2
Preventive effects of BBI & PAL-BBI on DMBA induced transformation of mouse mammary gland
in whole organ culture

Group	treatment			total glands No.	No. of NLAL/ total glands	transformed glands			toxicity*** (%)
	DMBA	BBI	PAL-BBI			No.	Incidence	Prevention**	
1	*0			40	2/40	2	5.00%		0.00%
2	2µg/ml			40	90/40	39	97.50%		0.00%
3	3µg/ml			20	40/16	16	80.00%		20.00%
4	4µg/ml			30	40/26	21	70.00%		13.33%
5		10µg/ml		20	1/20	1	5.00%		0.00%
6		20µg/ml		34	4/34	3	8.82%		0.00%
7		30µg/ml		20	3/19	1	5.00%		5.00%
8			20µg/ml	20	3/20	2	15.00%		0.00%
9	2µg/ml	10µg/ml		20	30/20	13	65.00%	33.67%	0.00%
10	2µg/ml	20µg/ml		30	14/28	10	33.33%	66.33%	6.67%
11	3µg/ml	20µg/ml		20	11/17	7	35.00%	56.25%	15.00%
12	4µg/ml	30µg/ml		20	6/12	4	20.00%	71.43%	40.00%
13	2µg/ml		20µg/ml	24	15/24	9	37.50%	61.22%	0.00%

*DMSO 10µl/5 ml (equal the volume of DMBA solvent) as a control.

** prevention=[1 - (incidence treated by DMBA+BBI or PAL-BBI)/incidence treated by DMBA only] %.

***toxicity means percentage of the glands without alveolar buds or light stain of the whole gland (dead glands).

resulted in an increase of the GI transit time of Pal-BBI as indicated in the time-dependence of the small and large intestine-associated radioactivity (Table 3).

3. Analysis of the stomach-associated BBI and Pal-BBI (Task 6).

Since the most noticeable difference between the orally administered BBI and Pal-BBI was the stomach retention, we further investigated the composition of the radioactivity in the stomach. In this study, stomachs of mice that were fed orally with either ^{125}I -BBI or ^{125}I -Pal-BBI, were cut to open and stomach-associated mucosal fluid was collected. The mucosal fluid was diluted with PBS and subsequently precipitated with 10% trichloroacetic acid (TCA). The TCA-precipitate fractions were considered as intact polypeptides and the TCA-soluble fractions as degradation products. As shown in Table 4, the difference between the intact polypeptide in the stomach of Pal-BBI fed mice and of BBI fed mice was even more striking than the total radioactivity. When expressed as the amount of intact polypeptide in the stomach, Pal-BBI was 34-, 17-, and 3-fold higher than BBI in 0.5, 1.5, and 3 hr, respectively. It must be emphasized here that BBI is an inhibitor of trypsin- and chymotrypsin-like proteases only. Therefore, it is not surprising that BBI can be degraded by pepsin in the stomach. Our results from the analysis of the stomach-associated Pal-BBI indicated that lipidization can increase the stability of a polypeptide in the GI tract. Whether the increase of gastric stability, in combination of the prolonged intestinal transit time, can be translated into a higher oral bioavailability of BBI or not, remains to be determined as the Task 7 in the coming year.

7. CONCLUSION

Even though the progress of this project was delayed by the contamination problem in the mammary gland cultures during the past year, we were able to obtain several important findings that, to our knowledge, have not been reported previously. We demonstrated in cultured mouse mammary glands that BBI, a polypeptide isolated from soybean, can significantly decrease the incidence of transformation induced by a chemical carcinogen, DMBA. We also demonstrated that the lipidized BBI, Pal-BBI, is at least equally potent as BBI in the chemoprevention of mammary gland transformation. Finally, we demonstrated that lipidized BBI is significantly more stable than BBI in the GI tract, an observation that may suggest a better oral absorption of Pal-BBI than of BBI. We feel confident that, within the next year, we will be able to achieve the goals of this project, i.e., to demonstrate that BBI is an active component in soybean for the prevention of breast cancer, and that Pal-BBI could be a better polypeptide than BBI when administered orally as a chemopreventive agent.

8. REFERENCES

1. St. Clair, W.H., Suppression of 3-methylcholanthrene-induced cellular transformation by timed administration of the Bowman-Birk protease inhibitor. *Carcinogenesis* 12:935-937 (1991).
2. Honeycutt, L., Wang, J., Ekrami, H. and Shen, W.C., Comparison of pharmacokinetic parameters of a polypeptide, the Bowman-Birk protease inhibitor (BBI), and its palmitic acid conjugate. *Pharm. Res.* 13:1373-1377 (1996).

Table 3
Biodistribution of orally administered BBI or Pal-BBI in Balb/c mice

Time (hr)	% total radioactivity (\pm SD, N=3)					
	BBI			Pal-BBI		
	0.5	1.5	3.0	0.5	1.5	3.0
Blood	4.6 (0.6)	4.3 (1.2)	6.6 (1.9)	4.1 (1.1)	6.9 (0.9)	6.2 (0.9)
Liver	1.1 (0.1)	0.7 (0.1)	1.1 (0.3)	1.0 (0.2)	1.3 (0.1)	1.1 (0.3)
Kidneys	0.5 (0.1)	0.4 (0.1)	0.7 (0.2)	0.4 (0.1)	0.7 (0.0)	0.7 (0.1)
Small Intestines	67.5 (8.1)	56.4 (7.4)	16.0 (22.2)	24.9 (4.5)	30.2 (3.1)	4.5 (1.1)
Large Intestines	0.3 (0.2)	2.5 (3.4)	20.0 (17.1)	0.4 (0.0)	1.1 (0.7)	18.6 (4.0)
Stomach	3.0 (0.9)	3.1 (0.1)	3.9 (1.3)	33.0 (7.4)	11.9 (6.0)	6.4 (2.0)

Table 4
Percentage of orally administered BBI or Pal-BBI as intact polypeptide in stomach of Balb/c mice

Time (hr)	% total dose as intact polypeptide in stomach (\pm SD, N=3)	
	BBI	Pal-BBI
0.5	0.8 (0.3)	27.5 (6.2)
1.5	0.4 (0.1)	6.9 (5.4)
3.0	0.5 (0.5)	1.5 (0.7)